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Biological effects of *Escherichia coli* lipopolysaccharide (LPS) *in vivo*

I. SELECTION IN THE MOUSE THYMUS OF KILLER AND HELPER CELLS

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Summary. In the present study we have investigated the biological effects on thymus lymphocytes resulting from *Escherichia coli* lipopolysaccharide (LPS) treatment in young adult mice. It has been established that LPS induces the following effects: (a) a dose-dependent reduction of thymus weight contemporaneous with a rise in the anti-LPS antibody response; (b) an increase of killer activity of thymus cells; (c) an enhancement of thymocytes helper activity; (d) a reduction of theta-positive cells in the thymus; (e) a cellular depletion in the thymus cortex. These data, indicating that LPS selects in the thymus a population of cells more efficient in expressing both killer and helper functions, are interpreted as caused by an increased rate of cortisol secretion induced by the LPS treatment.

INTRODUCTION

Several mitogenic substances have been used in mice to focus the biological functions of T and B lymphocytes in the immune response.

It has been shown that bacterial endotoxin or lipopolysaccharide (LPS) from *Escherichia coli* exerts a mitogenic effect inducing a marked B-lymphocyte proliferation *in vitro* (Andersson, Möller and Sjöberg, 1972). Furthermore, other studies suggest that T cells are not necessary in the anti-LPS antibody response (Möller and Michael, 1971; Manning, Reed and Jutilia, 1972; Veit and Michael, 1972), indicating that the only cell type involved in this response is the B lymphocyte. Recently some authors have suggested that LPS, besides directly stimulating B lymphocytes has also some biological effects on T cells, possibly influencing antigen-specific helper T-cell functions *in vitro* (Armerding and Katz, 1974; Kagnoff, Billings and Cohn, 1974).

Other investigators have also suggested that LPS *in vivo* may influence T lymphocytes, since it seems to affect immunological reactions such as allograft rejection (Al-Askari, Zweiman, Lawrence and Thomas, 1964), graft-versus-host reactions (Skopinska, 1972) and delayed hypersensitivity (Lagrange, Mackaness, Miller and Pardon, 1975; Lagrange and Mackaness, 1975).

The present study has been undertaken in order to elucidate the effects of LPS *in vivo* on thymus lymphocyte functions and on the morphology of the thymus.

The data presented here indicate that LPS induces functional modifications in the thymus cell

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compartment and are compatible with a selection of a resistant thymus cell population.

MATERIALS AND METHODS

Animals

Forty-five to 90-day-old (C57Bl/10 × DBA/2)F1 hybrids (briefly BDF1) and DBA/2 mice were used in the present study. In each experiment all animals were of the same sex.

Antigens

Lipopolysaccharide from *Escherichia coli* (LPS) O55:B5 Westphal method was purchased from Difco Laboratories, Detroit, Michigan (lot no. 604334) and was stored frozen at a concentration of 1·0 mg/ml in sterile, phosphate-buffered saline (PBS). Each dose of LPS was always given intraperitoneally in 0·1 ml of PBS. Sheep red blood cells (SRBC) were obtained from the Istituto Sieroterapico Sclavo, Siena, Italy.

Assay for anti-LPS antibody response

Groups of ten BDF1 mice aged 45 days were immunized intraperitoneally with a single injection of 0·1, 1, 10, 20, 40 or 80 μg of LPS. Plaque-forming cells (PFC) were determined in the spleens 4 days after immunization using SRBC coated with LPS as already described (Möller, 1965). Blood samples were collected at the time of killing, and the heat-inactivated sera (56° for 30 min) were tested for haemagglutination titres (Möller, 1965).

Irradiation

Irradiation conditions were described previously (Doria and Agarossi, 1968).

Cell preparations and transfer

Bone marrow cells from 90-day-old untreated BDF1 male mice were flushed from the femurs using cold TC medium 199 (Difco Laboratories, Detroit, Michigan). The cells were passed through a 26-gauge needle to disrupt cell clumps. Thymuses, from 45-day-old untreated or 20 μg LPS-treated mice, were removed under 4 × loupe magnification to avoid taking adjacent lymph nodes, and pressed through a 50-mesh stainless steel strainer in cold TC medium 199. Thymus cell suspensions were washed three times (1500 r.p.m. for 30 min). Bone marrow cell suspensions were centrifuged twice at 1500 r.p.m. for 10 min. Both cell preparations were filtered through a 20-μm nylon mesh, and nucleated cells were counted and mixed according to the protocol outlined in Table 1. Ninety-day-old BDF1 mice received a total X-ray dose of 900 rad. Each irradiated mouse received the appropriate number of cells intravenously in 1 ml of medium.

Assay for anti-SRBC antibody response in irradiated and reconstituted mice

At the time of cell transfer each irradiated and reconstituted recipient was injected intraperitoneally with 5×10^8 SRBC in 0·5 ml of PBS and boosted with the same dose of SRBC 3 days later. Six days after cell transfer the recipients were killed by exsanguination and the spleens removed for determination of the number of anti-SRBC PFC per spleen (Jerne, Nordin and Henry, 1963).

Cytotoxic test

Anti-theta serum was prepared in AKR mice by repeated injections of C3H thymus cells, and tested by the method of ^{51}Cr release using the technique originally described by Raff and Wortis (1970) to detect θ antigen on the surface of lymphocytes. The proportion of θ-bearing lymphocytes was determined using the cytotoxic test on sodium chromate (^{51}Cr) labelled thymus cells obtained from untreated and LPS-treated mice. Groups of 45-day-old BDF1 mice were injected with a single dose of 20 or 80 μg of LPS and were killed by exsanguination 4 days later. Thymuses were removed and immediately teased apart in Eagle's medium containing 0·1 per cent BSA (Armour). Cells were washed twice (1000 r.p.m. for 10 min) with Tris-buffered NH_4Cl to lyse red blood cells, filtered through a 20-μm nylon mesh and washed in Eagle's medium. The nucleated cell concentrations were adjusted to 2×10^7 cells/ml and then the suspensions were mixed with $Na_2{}^{51}CrO_4$ (Radiochemical Centre, Amersham, Bucks.) at a final concentration of 50 μCi/ml and incubated at 37° for 1 h. After incubation the cells were washed eight times with Eagle's medium, filtered through a 20-μm nylon mesh, counted and adjusted to a final concentration of 1×10^6 cells/ml in Eagle's medium. 0·1 ml Labelled cell suspensions was incubated with an equal volume of anti-theta serum or AKR brain-absorbed normal serum, both undiluted and diluted with PBS in a two-fold manner, at room temperature for 15 min. Thereafter, 0·1 ml of agar-absorbed

(Cohen and Schlesinger, 1970) guinea-pig complement (Sclavo, Siena, Italy) was added (final concentration, 1:10 in Eagle's medium) and the mixture incubated at 37° for 30 min. The reaction was stopped by addition of 1 ml of cold PBS. The tubes were centrifuged at 1500 r.p.m. for 10 min, and the supernatants decanted and counted in a well-type scintillation counter in duplicate. 0·1-ml Aliquots of each cell suspension were incubated as above with 1·2 ml of Triton, instead of serum, complement and PBS, to obtain complete lysis and ^{51}Cr release. The number of counts per minute (c.p.m.) was determined and the cytotoxic index calculated according to the formula:

(c.p.m. with anti-theta serum) − (c.p.m. with normal serum)/(c.p.m. with Triton) − (c.p.m. with normal serum) × 100.

Specificity of the anti-theta serum was inferred from complete disappearance of cytotoxicity after absorption with mouse brain. When target cells were incubated with normal serum and complement the ^{51}Cr release was within 10 per cent of the Triton control.

Graft-versus-host (GVH) mortality assay

Ninety-day-old BDF1 male mice were given a total-body-X ray dose of 500 rad. DBA/2 male mice aging 45 days were injected intraperitoneally with 20 μg of LPS in 0·1 ml of PBS 4 days before killing. 220 LPS-treated and 150 untreated DBA/2 mice of the same age were killed by exsanguination, and cell suspensions from thymuses were prepared in Eagle's medium as already described (see the section 'Cell preparations and transfer'). Cells were suspended at the appropriate concentration and injected intravenously, 2 h after irradiation, in three groups of BDF1 mice (forty recipients in each group). Each group received 1 ml of Eagle's medium alone or containing 20×10^6 DBA/2 nucleated thymus cells or 20×10^6 DBA/2 nucleated LPS-treated thymus cells.

Additional groups of 90-day-old DBA/2 mice (twenty recipients in each group) were given a total-body-X-ray dose of 500 rad and were injected intravenously 2 hours later with 1 ml of Eagle's medium alone, or containing 20×10^6 DBA/2 untreated nucleated thymus cells, or 20×10^6 DBA/2 LPS-treated nucleated thymus cells.

Morphological study

At the time of killing, body and thymus weights were recorded. Groups of five mice each were injected intraperitoneally with a single dose of 20 or 80 μg of LPS in 0·1 ml of PBS and killed serially 1, 2, 3, 4, 7 and 10 days later.

One additional group of six uninjected animals was kept as control. Thymuses were fixed in Carnoy's fluid, embedded in paraffin and sections were stained with haematoxylin and eosin, and methyl-green pyronin.

RESULTS

LPS has a dose-dependent effect on thymus weight and on the immune response to LPS

BDF1, aged 45 days, were injected with a single dose of LPS (0·1, 1 10 20 40 or 80 μg). The effects of treatment on thymus weight, number of anti-LPS PFC in the spleen and on haemagglutination titres are shown in Figs 1 and 2. These data demonstrate a progressive dose-dependent thymus weight decrease concomitant with an increase in the number of spleen PFC and in haemagglutination titre, reaching plateau values at the dose of 20 μg of LPS.

LPS enhances GVH activity of thymus cells in the mortality assay

Fig. 3 shows the results of an experiment designed to compare the GVH activity of untreated or LPS-

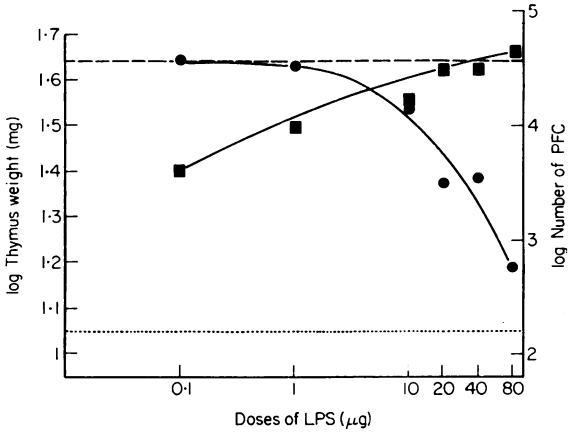

Figure 1. Thymus weights (●) and number of PFC (■) 4 days after a single injection of different doses of LPS. Each point represents the mean of at least ten observations. (– – –) Thymus weight of untreated mice. (· · ·) PFC of untreated mice.

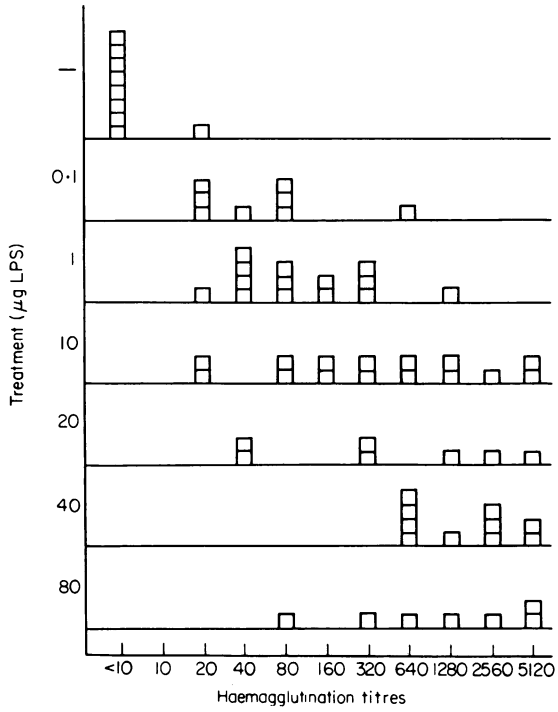

Figure 2. Haemagglutination titres 4 days after a single injection of different doses of LPS. Each square represents one observation.

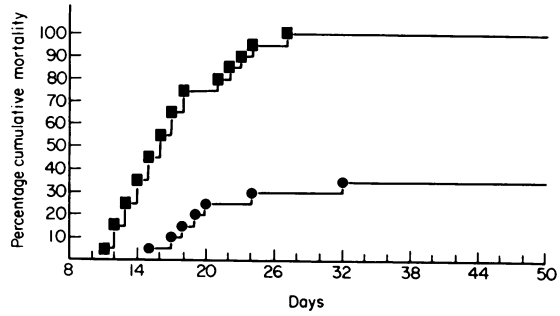

Figure 3. Cumulative mortality observed in sublethally irradiated BDF1 mice grafted with 20×10^6 DBA/2 thymus cells from untreated (●) or 20 μg LPS-treated (■) donors.

treated DBA/2 thymus cells injected in sublethally irradiated F1 hybrids. It can be seen that treatment *in vivo* with 20 μg of LPS 4 days before killing significantly enhances ($P<0{\cdot}01$) the GVH activity of thymus cells as compared to thymocytes from untreated donors. Actually, 100 per cent cumulative mortality was observed in F1 recipients of LPS-treated thymus cells on the 27th day, whereas recipients of untreated thymocytes reached a cumulative mortality of only 35 per cent on the 32nd day. The observation period for this experiment was 50 days. During this period no deaths occurred among irradiated F1 control mice which received no cells.

No mortality was observed among DBA/2 irradiated mice injected either with untreated or LPS-treated syngeneic thymus cells. Thus the difference in cumulative mortality observed between the two groups of F1 hybrids (Fig. 3) must be interpreted as caused by an enhanced GVH activity of the thymus cells from LPS-treated DBA/2 mice.

LPS enhances helper activity of thymus cells

Four days after injection with 20 μg of LPS, 45-day-old BDF1 mice were killed, their thymus cells prepared and transferred with normal bone marrow cells into lethally irradiated syngeneic recipients. Other groups of mice were grafted with normal thymus and bone marrow cells. Additional groups of animals received one of the following cell suspensions: LPS-treated thymus; untreated thymus; untreated bone marrow. At the time of cell transfer all mice were immunized intraperitoneally with SRBC and boosted 3 days later. The number of anti-SRBC PFC in the spleen was determined 6 days after cell transfer. As shown in Table 1, mice were given 40×10^6 bone marrow cells and a dose of thymus cells of 8×10^6 or 40×10^6. It is clear that 40×10^6 LPS-treated thymus cells were more effective in co-operating with bone marrow cells than untreated thymus cells. These findings were reproducible also in other experiments in which C3He/FeJ/Cas mice were used.

Influence of LPS injection on the sensitivity of thymus cells to anti-theta serum

To study the influence of LPS on the antigenic properties of thymus lymphocytes, we have determined the percentage of theta-bearing lymphocytes in the thymus of mice untreated or treated 4 days before killing with a single dose of 20 or 80 μg of LPS.

The mean cytotoxic index is presented in Fig. 4 as a function of the $\log_2$ final dilution of the antiserum in the reaction mixture before addition of the complement. Untreated control and 20 μg LPS-treated thymus cells had practically the same cytotoxic index. Plateau mean values were equally higher

Table 1. Helper activity of thymus cells from untreated or 20 μg LPS-treated donors in the response to SRBC in BDF1 mice*

Group†	Treatment of thymus donors	Number of thymus cells grafted ($\times 10^6$)	Number of marrow cells grafted ($\times 10^6$)	PFC/spleen‡
A	—	8	40	725
B	—	40	40	4600
C	—	40	—	125
D	—	—	40	52
E	LPS	8	40	885
F	LPS	40	40	13100
G	LPS	40	—	215
H	—	—	—	27

* All mice were lethally irradiated (900 rad) 1–2 h before cell transfer.

† Each group was composed of six mice.

‡ Spleen cells in each group were pooled.

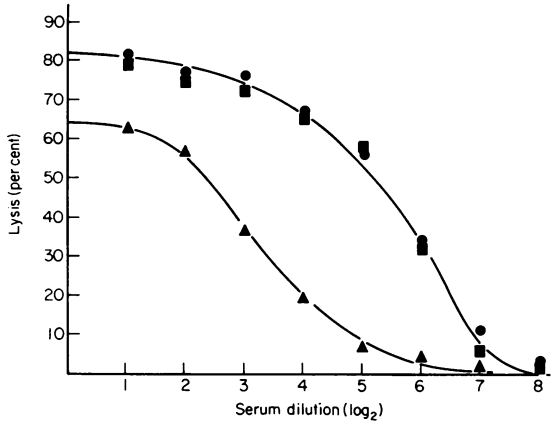

Figure 4. Cytotoxic index as a function of $\log_2$ final dilution of AKR anti-θ antiserum against BDF1 thymus cells. (●) Untreated controls; (■) 20 μg LPS; (▲) 80 μg LPS.

for mice either untreated or treated with 20 μg of LPS than for animals that had received 80 μg of LPS. Eighty micrograms of LPS treated cells were about four-fold less sensitive to anti-θ antibodies than untreated cells, as indicated by titres corresponding to 50 per cent of the plateau values.

Morphological observations

The thymus from 45-day-old untreated BDF1 mice as well as from mice of the same age injected with 20 or 80 μg of LPS and serially killed, as already indicated, was morphologically studied.

Pertinent gross morphological differences between the experimental groups and the uninjected control animals were limited to the weight of the thymus (Fig. 5). LPS treatment induces a thymus weight decrease related to the length of the interval between treatment and death of the animals. The greatest depression of thymus weight occurred on the 4th day following LPS treatment, reaching about 40 per cent of normal control values in mice injected with 20 μg and about 20 per cent in animals receiving 80 μg of LPS.

There was a good correlation between the change in thymus weight and the histological patterns of this tissue. As early as 24 h after the injection of 20 μg of LPS the thymic cortex was markedly reduced in thickness; in the subcapsular zone we observed very

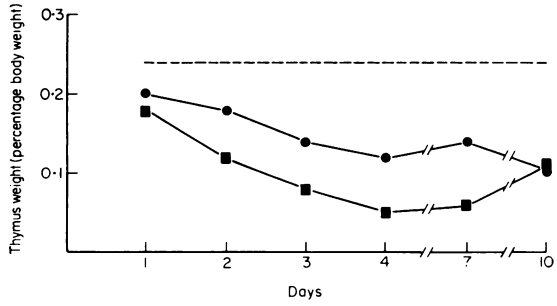

Figure 5. Effect of length of interval between LPS treatment on day 0 and killing on thymus weight expressed as percentage of body weight. (●) Twenty micrograms of LPS; (■) 80 μg of LPS.

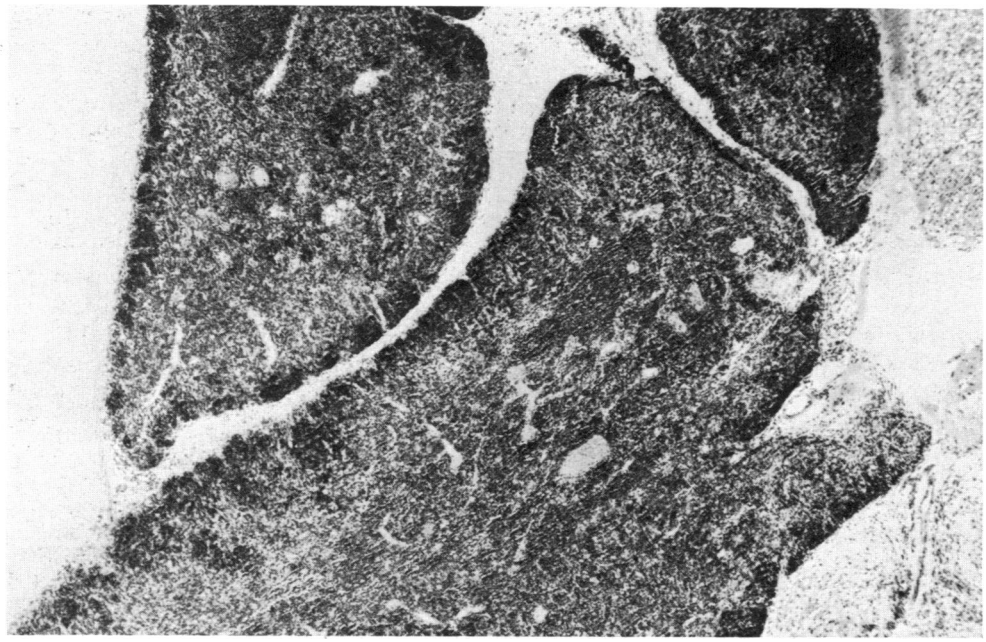

Figure 6. Thymus of a mouse treated with 20 μg of LPS and killed 2 days later. Note the reduction of the cortical lymphocyte. (H and E; magnification $\times 64$.)

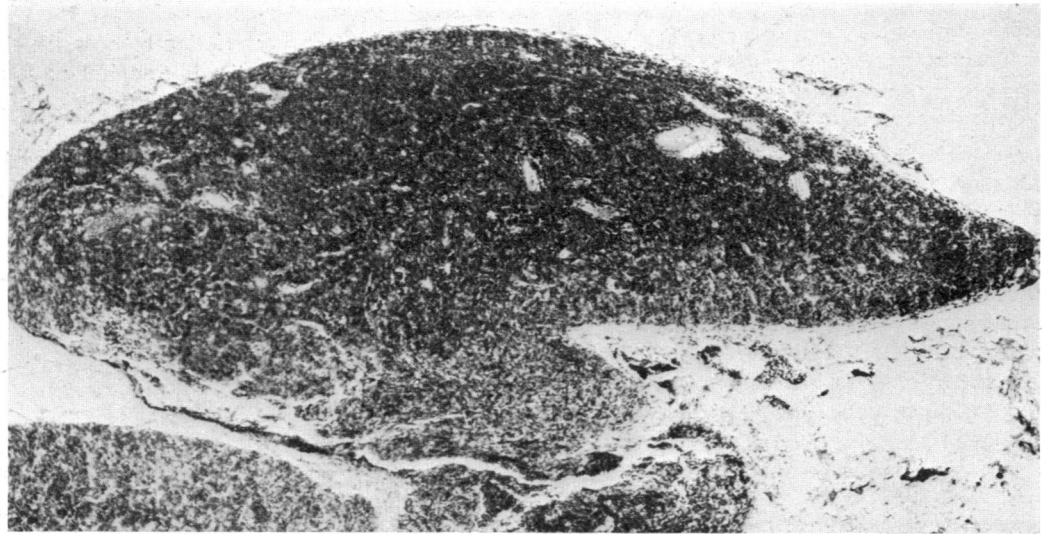

Figure 7. Thymus of a mouse treated with 80 μg of LPS and killed 2 days later. Small lymphocytes are mainly located in the medullary region (so called cortico-medullary inversion). (H and E; magnification $\times 64$.)

few lymphocytes and a remarkably low mitotic activity. Numerous macrophages containing cellular debris were scattered throughout the entire cortex.

On days 2 and 3 the thymic cortex was even more reduced in thickness than before (Fig. 6); however on day 3 in the subcapsular zone we could observe a thin layer of immature pyroninophilic cells with evident mitotic activity. On day 4 the thymus cortex, still being increased in thickness in many animals, was not yet completely normal. On days 7 and 10 after treatment, the thymus showed normal appearance.

The histological examination of the thymus of mice receiving 80 μg of LPS revealed the following microscopic patterns. As early as 48 h after treatment the thymus showed patterns of the so called cortico-medullary inversion. The cortex was almost totally depleted of lymphocytes whereas in the medulla lymphocytes were still present (Fig. 7). The above described histological pattern of the thymus remained practically unchanged till the 4th day following treatment. Beginning with the 7th day after LPS injection, morphologic patterns of thymic reconstitution were observed; 10 days following treatment the thymic cortex was still narrow and showed some irregularity in width as compared to untreated controls.

DISCUSSION

The present investigation has revealed the following effects: (1) LPS induces at the same time a marked dose-dependent decrease of the thymus weight and an increased anti-LPS antibody response; (2) LPS enhances killer and helper activities of thymus cells; (3) LPS decreases the theta-antigenicity of thymocytes; (4) LPS induces a marked lymphoid cell depletion in the thymus cortex.

The data presented above are compatible with the indication that LPS induces a selection in the thymus of a population of T lymphocytes more efficient in expressing both killer and helper activities.

It is reported that several *in vivo* treatments depressing the T-cell system are able to enhance the antibody response to B-dependent antigens. Kerbel and Eidinger (1972) observed that in mice early after adult thymectomy the anti-PPSIII response was increased as compared to sham-operated controls. Similarly Baker, Reed, Stashak, Amsbaugh and Prescott (1973) found a higher anti-PPSIII antibody response in mice pretreated with antilymphocyte serum. Also the induction of an acute GVH reaction enhances or suppresses the anti-PPSIII response depending on the dose and timing of the allogeneic cell treatment (Byfield, Christie and Howard, 1973). Under our experimental conditions we have observed evidence of thymus changes concomitant with an increased anti-LPS response. These observations may be explained by assuming that all the above described T-cell depressing treatments, as well as LPS, may eliminate suppressor T cells. Therefore B cells are not limited in expressing their antibody-forming activity, and the response to some T-independent antigens is increased.

LPS induces marked changes in thymus morphology, consisting mainly in necrosis and lymphocyte depletion in the cortex, whereas the medulla seems not to be affected. As previously demonstrated, less mature thymocytes are mainly located in the thymus cortex (Stobo, 1972). Therefore our findings, showing that LPS treatment markedly affects the thymus cortex, may indicate that an enrichment in more mature lymphocytes occurs in the thymus.

In the present experiment we have examined the effect of LPS treatment on thymocytes tested in the GVH assay. Our data indicate that thymocytes surviving LPS treatment are much more efficient in killing sublethally irradiated recipients than thymocytes from untreated controls, thus indicating that LPS favours an enrichment of killer T cells within the thymus.

In the present paper we have also shown that thymus cells from mice treated with LPS are more efficient than untreated thymocytes in the reconstitution of the antibody response to SRBC in lethally irradiated recipients. These data can be explained by postulating that LPS also selects in the thymus a population of helper cells. As previously proposed by Cohen and Claman (1971) for hydrocortisone acetate, LPS may enrich the thymus in killer and helper cells which are cortisone- and LPS-resistant.

In the cytotoxic test thymus cells from mice treated with 20 μg of LPS displayed the same θ antigenicity as thymocytes from untreated mice. However, following injection of 80 μg of LPS, the plateau value and the shape of the curve indicate that thymus cells resistant to this dose of LPS possess lower concentration of theta antigen, a property of more mature T cells (Raff and Cantor, 1971). It should be pointed out that the cytotoxic test might be less sensitive than functional tests in detecting cellular changes induced by different doses of LPS.

In conclusion, the present data show that LPS induces *in vivo* several biological effects on the thymus cells. These effects may be interpreted either as caused by a direct action of LPS on thymus cells or through a release of endogenous cortisol since LPS is known to be a stressing agent (Westphal, 1975). According to the results described in the accompanying paper (Adorini, Ruco, Uccini, Soravito De Franceschi, Baroni and Doria, 1976) the latter explanation seems to be more likely. The demonstration that the action of LPS on T cells within the thymus is mediated through cortisol secretion (Adorini *et al.*, 1976) adds further interest to our observations, since it suggests the possibility of investigating the effects of corticosteroid hormones on the immune response using endogenous cortisol at physiological levels. Furthermore, the present findings indicate that LPS represents a useful tool which can be of help in further dissecting the various components of the immune response.

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